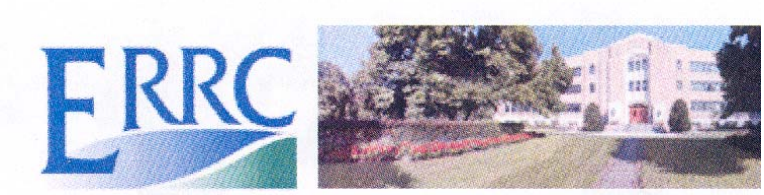

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Title: Effect of Bacterial Protein Extraction Reagent Combined with Sodium Azide on the Detection of *Escherichia coli* O157:H7 Using Immunomagnetic-Electrochemiluminescence

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EFFECT OF BACTERIAL PROTEIN EXTRACTION REAGENT COMBINED WITH SODIUM AZIDE ON THE DETECTION OF *ESCHERICHIA COLI* O157:H7 USING IMMUNOMAGNETIC-ELECTROCHEMILUMINESCENCE[1]

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ABSTRACT

Immunomagnetic capture combined with electrochemiluminescent detection (IM-ECL) is a rapid and sensitive method for the detection of pathogenic microorganisms from food. Prior to ECL detection enriched samples are often heated to reduce the viability of pathogens. In this work, a number of cell-lysis reagents and biocides were examined as alternatives to the heat treatment step to decrease bacterial viability in enriched samples as well as to improve the IM-ECL detection of Escherichia coli *O157:H7. A combination of Bacterial Protein Extraction Reagent (B-PER™), a nonionic detergent, and sodium azide (NaN_3) decreased bacterial viability to below the level of detection (< 10 cells/mL) in ground beef enrichments and resulted in a 6-fold increase in the sensitivity of IM-ECL detection of* E. coli *O157:H7.*

INTRODUCTION

With increased interest in developing rapid detection techniques to prevent foodborne illnesses, researchers have explored alternative methods to

[1] Mention of brand names does not constitute endorsement by U.S. Department of Agriculture over others of a similar nature not mentioned.

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conventional, direct culturing of microbial pathogens from food matrices. One such detection system which has gained considerable attention in recent years is the immunomagnetic capture-electrochemiluminescence detection (IM-ECL) (Crawford *et al.* 2000; Gatto-Menking *et al.* 1995; Yu and Bruno 1995, 1996). The IM-ECL method utilizes the combination of a immunomagnetic bead-based capture of minute amounts of target molecules or organisms from complex mixtures (Benett *et al.* 1996; Fratimico *et al.* 1992; Safarik *et al.* 1995; Tu *et al.* 1999) followed by electrochemiluminescence, a widely used research tool in clinical diagnosis (Blackburn *et al.* 1991; Blohm *et al.* 1996; Deaver 1995; Jameison *et al.* 1996; Stern *et al.* 1995) to detect the captured molecules or organisms.

Previously, Crawford *et al.* (2000) developed an IM-ECL method to detect *E. coli* O157:H7 in ground beef using a commercially available instrument, the ORIGEN analyzer (IGEN, International, Gaithersburg, MD). With its high sensitivity and rapid detection capability, it is an attractive method for detecting *E. coli* O157:H7 in food samples. This method was adapted into IGEN's proprietary ORIGEN technology to develop commercial diagnostic test kits for *E. coli* O157 and other foodborne pathogens (PathIGEN™). In January 2003, IGEN's PathIGEN™ *E. coli* O157 detection kit received AOAC approval (Certificate No. 010301).

To comply with the biosafety requirements of the regulatory agencies, Crawford *et al.* (2000) incorporated a heat treatment step to minimize the creation of aerosols of live pathogens. Samples were then filtered to avoid subsequent clogging of the ORIGEN analyzer by particulate material formed during the heat treatment. While heat treatment induced about a three-fold increase in the sensitivity, the heating and filtration steps prolonged the assay time significantly.

We investigated the use of a variety of cell-lysis reagents (detergents) and chemical biocides as possible alternatives to the heat-killing step. A combination of Bacterial Protein Extraction Reagent (B-PER™) (Pierce, Rockford, IL), a proprietary, mild, nonionic detergent formulation causing bacterial cell lysis, and sodium azide (NaN_3), a respiratory inhibitor commonly used as a preservative, was the most effective treatment among the ones we tested and results from these experiments are presented here.

MATERIALS AND METHODS

Bacterial Growth and Enumeration

E. coli O157:H7 strain B 1409 (Center for Disease Control & Prevention, Atlanta, GA) was grown in 25 mL of brain heart infusion broth (BHI; Difco,

Detroit, MI) at 37C for 18 h at 160 RPM. The number of bacterial cells in the cultures was determined by diluting the culture 100-fold with phosphate buffered saline (PBS) and counting with a Petroff-Hausser Chamber (Hausser Scientific, Horsham, PA) (Tu *et al.* 1999).

To determine the number of colony forming units (cfu), the cultures were serially diluted in PBS, and 50 μL of dilutions of approximately 10^2, 10^3 and 10^4 cells/mL were plated on Brain Heart Infusion Agar (BHIA) in triplicate using a Spiroplater (Spiral Biotech, Bethesda, MD) in the Spiral Mode. After incubating at 37C overnight, the colonies were counted. The cultures were also diluted to about 10^6 cells/mL with either PBS or beef broth to use as inocula in subsequent experiments.

Beef Broth Samples (BBS)

Beef broth samples (BBS) were prepared as follows: Twenty five gram portions of ground beef (85% lean, from a local supermarket) were incubated for 18 h in stomacher bags (Tekmar Co., Cincinnati, OH) with 225 mL of Modified EC (Difco, Detroit, MI) containing 20 mg/L of sodium novobiocin; Sigma, St. Louis, MO) at 37C at 160 RPM. Samples were mixed in a Stomacher 400 (Seward Ltd., London) for 2 min prior to incubation. The samples were withdrawn from the bag between the filter and the side of the bag where ground beef was not present, and filtered into tubes through 300 μm-pore disposable polypropylene filters (Fisher, Pittsburgh, PA). These samples were aliquoted into sterile microcentrifuge tubes and stored at -20C for use later. Where indicated, fresh BBS was used. Fresh BBS was prepared as described above except that it was not frozen and it was used the day it was prepared.

To determine the bacterial population of BBS, 10-fold dilutions of BBS were made with PBS, and dilutions from 10^2-10^6 were plated on three plates each of BHIA and two selective/differential media for *E. coli* O157:H7, Biosynth Culture Medium (BCM^R) (Biosynth International, Naperville, IL) and Rainbow agar (RBA) (Biolog, Hayward, CA) using the Spiral Mode (100 μL/plate) or Lawn Mode (20 μL/plate) of the Spiroplater (Spiral Biotech). The difference between the number of cfu's on the selective/differential media and BHIA allowed us to differentiate *E. coli* O157:H7 in inoculated BBS as opposed to bacteria indigenous to the BBS.

Electrochemiluminescence (ECL) Analysis

The ORIGEN Analyzer (Version 2.7) (IGEN International, Gaithersburg, MD) was used for ECL-detection. Unless otherwise specified, triplicate, 1-mL or 1.02-mL samples were analyzed in Elkay disposable polypropylene tubes (12 $\times$ 75 mm) (Thomas Scientific, Swedesborough, NJ). The samples were incubated with 50 μL of antibody-tag mixture consisting of anti-*E. coli* O157

immunomagnetic beads (2.5 μL/sample) (Dynal A.S., Oslo, Norway) and ruthenium labeled anti-*E. coli* O157 (100 ng/sample) in PBS blocking buffer containing 2% goat serum (Rockland, Gilbertsville, PA) and 2% Tween 20 (Sigma, St. Louis, MO) in sample tubes with rigorous shaking on a carousal of the ORIGEN instrument for 1 h prior to analysis. A detailed description of the procedure including labeling of antibodies and preparation of other necessary components was reported by Crawford *et al.* (2000).

Cell-lysis Reagents and Biocides

In an attempt to find a suitable cell-lysis reagent, several commercial products were tested. The cell-lysis reagents, BIOPROB Extractant, a proprietary solution from Hughes Whitlock Ltd. (Madison, WI), Bacterial Protein Extraction Reagent (B-PER) and Yeast Protein Extraction Reagent (Y-PER) (Pierce, Rockford, IL) were used in combination with the biocide preservatives, Kathon CG/ICP II, ProClin 300 (Supelco, Bellefonte, PA) and sodium azide (NaN_3; Sigma Chemical Co., St. Louis, MO) in preliminary experiments.

To examine the effect of B-PER (Pierce, Rockford, IL), a nonionic liquid detergent in phosphate buffer, and NaN_3 on the ECL detection of *E. coli* O157:H7, three separate experiments were run with each being repeated at least three times.

ECL Detection of *E. coli* O157:H7 in Pure Culture Using B-PER

To compare the ECL response of B-PER treated samples to those treated with PBS at various concentrations of *E. coli* O157:H7, an overnight cultures (approximately 10^9 cells/mL; cells were counted for each experiment using a Petroff-Hausser Chamber as described above) were diluted to 2×10^5 cells/mL in PBS or B-PER. The cell suspensions were further diluted by two-fold serial dilution to 1,562 cells/mL in either B-PER or PBS. One milliliter of each dilution was distributed into three tubes. Samples with B-PER or PBS without cells were used as controls. The samples with B-PER were incubated for 10 min at room temperature (RT) to provide time for the cells to lyse prior to adding 50 μL of antibody-tag mixture and placing on the ORIGEN analyzer. The samples diluted in PBS were placed on the carrousel, followed by the B-PER treated samples, from the lowest to the highest B-PER concentration, with cell-free sample controls being the first tubes of each set. The samples were shaken for an hour and the ECL analysis was performed as described previously (Crawford *et al.* 2000).

Effect of B-PER Concentration on ECL Detection of *E. coli* O157:H7

One hundred microliters of thawed BBS was combined with 300, 400, 600, 750 or 900 μL of B-PER and the sample volume of each tube was adjusted to 1 mL with PBS. Six sample tubes were prepared for each concentration of B-PER. For each B-PER concentration, 20 μL of PBS was added to three of the samples (noninoculated control) while 20 μL of PBS containing 5×10^6 cells of *E. coli* O157:H7 (to give the final cell concentration of 10^5 cells/mL) was added to other three samples. All samples with B-PER were incubated for 10 min at RT prior to adding 50 μL of antibody-tag mixture and placing on the ORIGEN analyzer. The samples were shaken for an hour and the ECL analysis performed as described above.

Heat treated samples were prepared by combining 100 μL of BBS, 900 μL of PBS and 20 μL of PBS containing 5×10^6 cells of *E. coli* O157:H7. Six samples were prepared. Three of these samples were heated for 10 min at 100C on a heating block (Crawford *et al.* 2000) prior to analysis (heat-killed cells) and three samples were not heat treated (live cells).

Combined Effect of B-PER and NaN_3 on Bacterial Viability and ECL Detection of *E. coli* O157:H7

Thawed or freshly prepared BBS was mixed either with B-PER or PBS at the ratio of 1:9. Nine hundred microliters of the above mixture was combined with 20 μL of PBS containing 5×10^6 *E. coli* O157:H7 cells and 100 μL of either ultra pure water or a stock solutions of NaN_3 (Sigma Chemical Co.) in sterile 18 MΩ-cm water (NANApure, Barnstead/Thermolyne, Dubuque, IA) to give final concentrations of 0.01, 0.05, 0.1 and 0.5% NaN_3. All samples and noninoculated BBS control samples for each treatment were prepared in triplicate. Samples were incubated for 10 min at RT prior to adding 50 μL of antibody-tag mixture and placing on the ORIGEN analyzer for ECL detection. For estimating the number of cfu, a similar set of treatments were prepared and the samples were plated (100 μL/plate) in duplicate on BHIA using the Spiroplater.

RESULTS AND DISCUSSION

Bacterial Populations in BBS

Freezing BBS resulted in approximately a thousand-fold decrease in indigenous bacterial population (data not shown). When freshly prepared BBS was plated on BHIA for viable cell determinations, the indigenous bacterial population was 10^8 - 10^9 cfu/mL; in samples stored at -20C for 3 weeks, it was

10^5-10^6 cfu/mL. Thus, when the total bacterial population of the samples was determined by plating on BHIA, the added *E. coli* O157:H7 (10^5 cells per sample) contributed little to the total bacterial population of fresh BBS; but, the added *E. coli* resulted in a measurable increase in the total bacterial population of frozen BBS. By plating on BCM or RBA plates, the distinct colony morphology of *E. coli* O157:H7 on the selective/differential media allowed a determination of the number of *E. coli* O157:H7 recovered compared to the indigenous population observed on BHIA. Based on experiments using *E. coli* O157:H7 B 1049 in pure culture and mixed with frozen/thawed BBS, the recovery of *E. coli* O157:H7 was 70% on RBA and 50% on BCM.

Cell-lysis Reagents and Biocides

Of the reagents tested (BIOPROBE Extractant, B-PER, Y-PER, Kathon, Proclin 300, and NaN_3) only B-PER and NaN_3 significantly reduced cell viability while improving the ECL detection of *E. coli* O157:H7 (data not shown). Although BIOPROBE Extractant was effective in reducing the cell viability, this treatment compromised the ECL detection of *E. coli* O157:H7 (data not shown). B-PER or a combination of B-PER and NaN_3 reduced the cell viability and increased the ECL values significantly (see below).

ECL Detection of *E. coli* O157:H7 in Pure Culture Using B-PER

Initial experiments to evaluate the effect of B-PER treatment on the ECL detection of *E. coli* O157:H7 at various cell concentrations were conducted with pure cultures. The ECL detection of both live cells (in PBS) and B-PER treated cells was linear in the range of 10^3 - 10^5 cells/mL and B-PER treatment resulted in a 6 to 11-fold increase in ECL response (Fig. 1). Even at the lowest concentration of cells the ECL signal-to-noise (response to background) ratios were 3.2 and 4.7 for the live and B-PER treated cells, respectively, and increased with increasing cell concentrations (Fig. 1).

Effect of B-PER Concentration on ECL Detection of *E. coli* O157:H7 in BBS

Since B-PER treatment significantly increased the ECL response (Fig. 1), the optimum concentration of B-PER for ECL detection of *E. coli* O157:H7 in BBS was determined. It was reported previously (Crawford *et al.* 2000) that heat treatment of *E. coli* O157:H7 enrichment cultures resulted in an approximately 3-fold increase in ECL response. A similar response to heat treatment is reported here (Table 1). Addition of as little as 30% B-PER to *E. coli* O157:H7 in BBS resulted in an increase in ECL response similar to that of the heat treatment (Table 1). Higher concentrations of B-PER resulted in an even greater

enhancement of the ECL response with 75% B-PER giving the highest signal to noise ratio (Table 1).

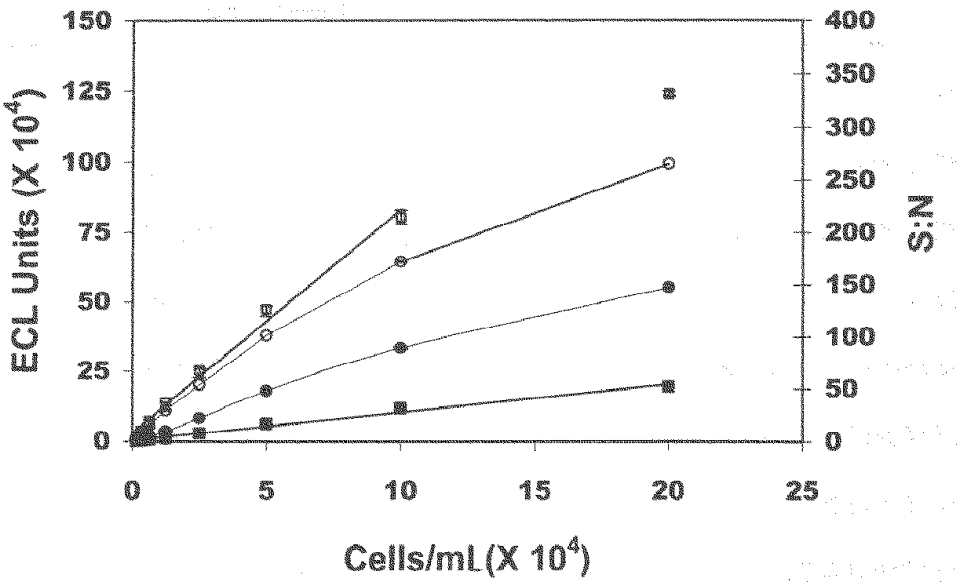


FIG. 1. THE EFFECT OF B-PER TREATMENT ON THE ECL DETECTION OF PURE CULTURES OF *E. COLI* O157:H7

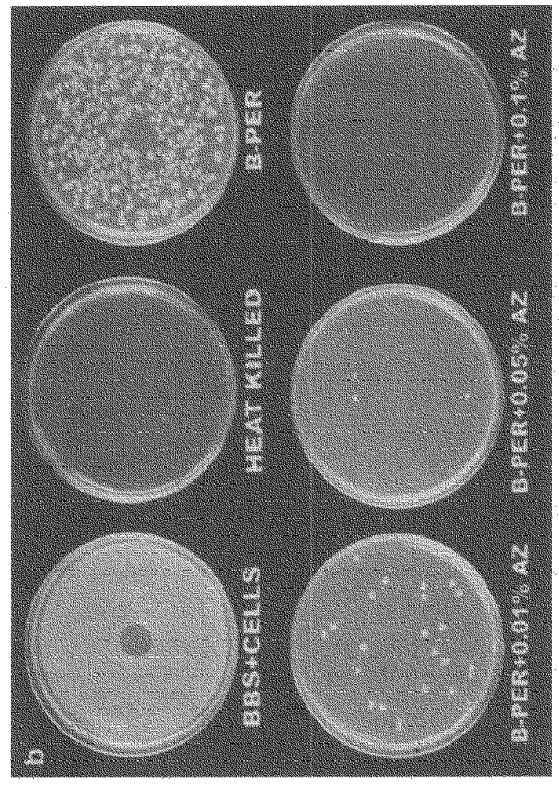
ECL values (squares) and signal-to-noise ratio (S:N, circles) for detection of *E. coli* O157:H7 in B-PER (open symbols) and PBS (solid symbols) at various cell concentrations (1,562; 3,125; 6,250; 12,500; 25,000; 50,000; 100,000; 200,000 cells/mL). A line of best fit is drawn through the linear range for ECL detection.

When samples prepared using frozen BBS were plated on BHIA to determine cell viability, heat treatment was found to completely eliminate the population of culturable cells (Fig. 2b). While all concentrations of B-PER significantly decreased the bacterial population, not even 90% B-PER completely eliminated all culturable cells (Fig. 2b). However, for samples prepared with fresh BBS, the number of cfu's after B-PER treatment was considerably higher for samples prepared from frozen BBS. Moreover, even the heat treatment did not completely eliminate the population of cells in samples prepared using fresh BBS (data not shown). The higher number of cells surviving in treated samples containing fresh BBS as compared to samples containing frozen BBS is most likely due to the higher number of cells in the fresh BBS and the cell death and sub-lethal injury caused by freezing the BBS.

TABLE 1.
EFFECT OF B-PER CONCENTRATION ON ECL DETECTION OF *E. COLI* O157:H7 IN FROZEN/THAWED BBS

[a]Sample treatments	[b]ECL units (mean ± SD)	[c]S:N
BBS	4,179 ± 774	
BBS+300µl B-PER	4,690 ± 653	
BBS+300µl B-PER+*E. coli*	761,755 ± 25,529	162
BBS+400µl B-PER	6,123 ± 420	
BBS+400µl B-PER+ *E. coli*	893,587 ± 26,807	146
BBS+600µl B-PER	6,877 ± 267	
BBS+600µl B-PER+ *E. coli*	1,127,355 ± 33,024	164
BBS+750µl B-PER	6,383 ± 368	
BBS+750µl B-PER+ *E. coli*	1,223,035 ± 59,368	192
BBS+900µl B-PER	8,752 ± 447	
BBS+900µl B-PER+ *E. coli*	1,195,520 ± 47,281	137
BBS+LIVE *E. coli*	256,603 ± 15,291	
BBS+HEAT KILLED *E. coli*	781,888 ± 20,687	

[a] Samples consisted of 100 µL BBS, 10^5 cells of *E. coli* O157:H7 in 20 µL of PBS and specified amounts of B-PER brought to a final volume of 1.02 mL with PBS.
[b] Average ECL with standard deviation of 3 samples per treatment
[c] Signal to noise ratio for each concentration of B-PER(BBS+B-PER+*E. coli*/BBS+B-PER)



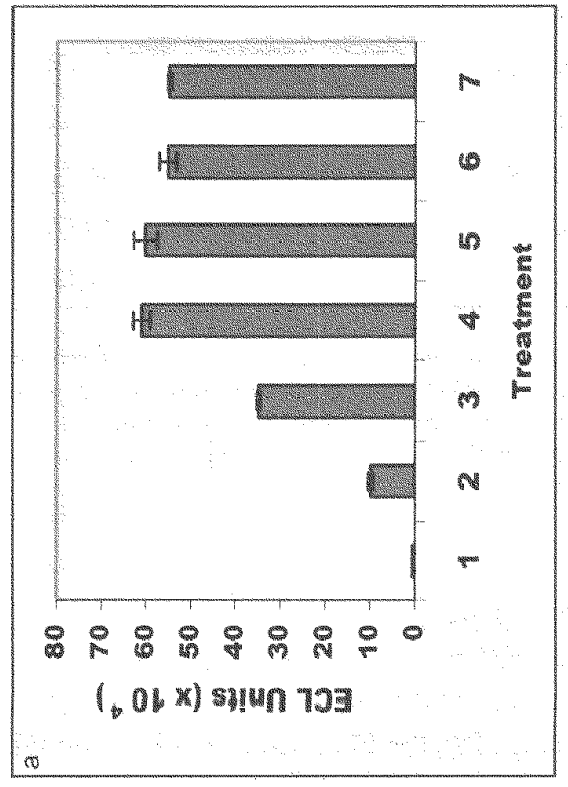


FIG. 2. EFFECT OF B-PER AND NaN_3 ON ECL DETECTION OF *E. COLI* O157:H7 AND BACTERIAL VIABILITY
(a) Average ECL value for treatments: 1, frozen/thawed BBS without treatment and (2-7) frozen/thawed BBS plus 10^5 *E. coli* O157:H7; 2, no treatment; 3, heat-killed; 4, B-PER; 5, B-PER plus 0.01% NaN_3; 6, B-PER plus 0.05% NaN_3; and 7, B-PER plus 0.1% NaN_3.
(b) Growth of BBS plus 10^5 *E. coli* O157:H7 on BHIA plates (treatments 2-7, from a).

Combined Effect of B-PER and NaN_3 on Bacterial Viability and ECL Detection of *E. coli* O157:H7 in BBS

The fact that neither 90% B-PER or heat treatment completely eliminated the bacterial population in fresh BBS led us to investigate additional alternatives to reduce the number of cells in BBS prior to ECL analysis. When B-PER was used in combination with NaN_3, more effective reduction in cell viability was obtained with both fresh and frozen BBS without sacrificing the enhancement in ECL response (Table 2 and Fig. 2).

TABLE 2.
CUMULATIVE EFFECT OF NaN_3 AND B-PER ON *E. COLI* O157:H7 IN FRESH BBS

[a]Sample treatments	[b]ECL units (mean ± SD)	Total Bacteria [c](cfu/ml)
BBS+ PBS	264,983 ± 10,005	[d]TNTC
BBS + B-PER	995,234 ± 45,801	TNTC
BBS + PBS + 0.01% NaN_3	262,068 ± 26,945	TNTC
BBS + B-PER + 0.01% NaN_3	957,727 ± 40,700	TNTC
BBS + PBS + 0.05% NaN_3	283,477 ± 38,628	TNTC
BBS + B-PER + 0.05% NaN_3	865,688 ± 54,886	765 ± 10
BBS + PBS + 0.1% NaN_3	282,194 ± 15,041	TNTC
BBS + B-PER + 0.1% NaN_3	902,615 ± 46,791	10 ± 0.75
BBS + PBS + 0.5% NaN_3	322,558 ± 10,333	53 ± 1.75
BBS + B-PER + 0.5% NaN_3	840,099 ± 41,185	<10

[a] Eighty percent B-PER final concentration; samples consisted of 10^5 cells of *E. coli* O157:H7 in 20 μL PBS, 900 μL of BBS:PBS (1:9) or BBS:B-PER (1:9) and 100 μL of NaN_3 or water
[b] Average ECL with standard deviation of 3 samples per treatment
[c] Colony forming units per milliliter from 100 μL of sample plated on BHI agar (average of 6).
[d] Too numerous to count

The effects of B-PER combined with NaN_3 on the ECL detection of *E. coli* O157:H7 and bacterial cell survival in frozen BBS are shown in Fig. 2. Though the addition of NaN_3 decreased the total number of cells recovered from B-PER-treated BBS (Fig. 2b), the ECL detection of *E. coli* O157:H7 in frozen

BBS was not affected (Fig. 2a). Once again the ECL detection of *E. coli* O157:H7 was 3-fold higher in heat treated samples compared to untreated samples. The addition of B-PER enhanced the ECL detection of *E. coli* O157:H7 6-fold compared to the untreated samples (Fig. 2a) and the enhancement was not diminished by the addition of NaN_3. Although 80% B-PER significantly reduced the number of bacteria in the sample, the addition of NaN_3 augmented the bacterial killing effect (Fig. 2b). Only the heat treatment as well as the combination of 80% B-PER and 0.1% NaN_3 reduced the number of cfu below the level of detection (< 10 cells/mL).

In fresh BBS inoculated with *E. coli* O157:H7, the combination of 80% B-PER with 0.05% and 0.1% NaN_3 reduced the total bacterial population from 10^8 - 10^9 cfu/mL, to about 10^3 and 10^1 cfu/mL, respectively, while still enhancing the ECL response at least three-fold as compared to untreated samples (Table 2). A combination of 80% B-PER and 0.5% NaN_3 was necessary to reduce the number of surviving bacteria to below the limits of detection (< 10 cells/mL). In the absence of B-PER, NaN_3 did not increase the ECL response to *E. coli* O157:H7 nor was the bacterial population reduced sufficiently to allow quantification, except at the highest concentration (0.5%) tested.

CONCLUSION

This study was undertaken to investigate alternative methods for the treatment of ground beef enrichment samples prior to ECL detection of *E. coli* O157:H7. When developing a method for the ECL detection of *E. coli* O157:H7 using the commercially available ORIGEN analyzer, Crawford *et al.* (2000) introduced a heat treatment step to reduce the likelihood of generating pathogenic aerosols. This step was very efficient in killing the bacteria and increased the sensitivity of ECL detection approximately 3-fold; but, necessitated the filtering of the samples to remove particulate matter generated during heating. In this study, treatment of ground beef enrichment samples with 80% B-PER and 0.1% NaN_3 prior to ECL detection using the ORIGEN analyzer significantly reduced the number of culturable bacteria in the samples (8 log reduction), increased the sensitivity of the assay by 3.4 fold, and decreased the assay time by eliminating the need for heat treatment and filtration steps. Thus, B-PER/NaN_3 treatment is an appealing alternative to heat treatment during sample preparation prior to detecting foodborne pathogens using ORIGEN instrument.

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